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(54) Title: MATRIX OF ANTIBODY-COATED SPOTS FOR DETERMINATION OF ANTIGENS

## (57) Abstract

Novel immunoassay device and method for the determination of antigenic substances. The device essentially comprises a pattern or array of minute antibody-coated spots on the surface of a support. The array of antibody-coated spots is preferably in the form of a rectangular matrix. Each antibody-coated spot is made up of antibodies of a different and distinct specificity. A large number of different antibody-coated spots can be assembled on a very small portion of the surface of the support. The spots serve as tiny, specific immunoadsorbents of cells. The expression of particula surface antigen by cells may be detected by determining to which antibody-coated spot the cells bind.

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-1-

MATRIX OF ANTIBODY-COATED SPOTS FOR  
DETERMINATION OF ANTIGENS

Description

Technical Field

5 This invention is in the fields of cell biology and immunology, and particularly relates to a novel immunoassay device and method for the determination of antigenic substances.

Background

10 Antigens on the surface of cells can be detected by their specific reactivity with antibodies in a number of ways. The most commonly employed methods are immunofluorescence staining, radiobinding assays and complement-mediated cell lysis. In each method,  
15 the reactivity of cells with a particular antibody to a cell-surface antigen is determined separately, that is, the cells are incubated separately with each antibody to be tested. For example, in a complement-mediated cell lysis assay using micro-  
20 titer plates, cells are incubated with antibody separately in each well of the microtiter plate. Usually, the microtiter plate is purchased with the desired antibodies frozen in the wells. After cells are added to each well and allowed to interact with  
25 antibody, complement is added along with a dye. Cells having antigens to which the antibodies bind are lysed and the lysed cells take up the stain. In this way cells to which antibodies have bound may be

-2-

determined by visually inspecting the microtiter plate.

5 Frequently the number of individual tests in certain determinations can be very large. For instance, in tissue typing where the cell surface  
10 allo-antigens encoded by the gene loci of the histocompatibility complex are determined, at least 80 individual tests are required to determine the allotypes of the HLA-A, -B, -C and -DR transplanta-  
15 tion antigens of a person. Such a large number of determinations entails a great deal of repetitive pipetting. Repetitive pipetting is undesirable because it is time consuming and because it in-  
creases the chance of error. Also a large number of  
20 determinations generally demand a large amount of sample and reagents which oftentimes are limited in supply because they are difficult or costly to obtain.

#### Disclosure of the Invention

20 This invention exploits the phenomenon of immunoadherence to arrive at a simple yet powerful technique for the determination of specific anti-  
genic substances on the surface of cells.

25 In its broadest sense this invention consti- tutes a novel immunoassay device and method for detecting antigenic substances. The invention is especially useful for determining the presence of  
30 particular antigens on the surface of cells, but is not limited to this use alone, as it may be employed for determining antigens in solution as well.

-3-

The device essentially comprises a pattern or array of minute antibody-coated spots on the surface of a support. Each antibody-coated spot is made up of antibodies of a different and distinct specificity. The spots serve as tiny, specific immuno-adsorbents of cells. The expression of particular surface antigen by cells may be detected by determining to which antibody-coated spot the cells bind. A large number of different antibody-coated spots can be assembled on a very small portion of the surface of the support.

The invention represents a major simplification of existing immunocytological and immunochemical methodology in that it eliminates the need for repetitive pipetting when a large number of individual tests for different antigens are performed such as in tissue typing. Other significant advantages of the invention are that all tests with antibodies may be performed simultaneously in one location, only a single application of sample is required as opposed to the repetitious pipetting of sample into a large number of separate wells or tubes, and very small amounts of sample and reagents are consumed. Also this invention eliminates the need for costly reagents such as complement which is required, as its name implies, in the complement-mediated cytolysis technique.



-4-

### Brief Description of the Drawings

Figure 1 shows the binding of mononuclear cells from an individual with allotype HLA-A2 to anti-HLA-A2 antibody spots on glass surface. Figure 1a shows the binding of the mononuclear cells to an anti-HLA-A2 antibody spot of 1 mm in diameter, Figure 1b shows binding to a spot of 0.5 mm in diameter, and Figure 1c shows binding to a spot of 0.25 mm in diameter.

Figure 2 shows the binding of mononuclear cells to a 10 x 10 spot matrix of anti-HLA-A2 antibody-coated spots.

Figure 3 shows the binding of thymocytes from AKR/J, BALB/c, and their F1 to antibody matrixes containing anti-Lyt 2.1 and anti-Lyt 2.2.

Figure 3a shows schematically the arrangements of the two antibodies in the matrix. Coating of antibodies, application of cell samples, and photography were described in the Examples.

Figure 3b is the cell binding result with thymocytes of (AKR/J x BALB/c)F1.

Figure 3c is the cell binding result with thymocytes of AKR/J.

Figure 3d is the cell binding result with thymocytes of BALB/c.

### Best Mode for Carrying Out the Invention

The immunoassay device of this invention comprises a support which has on its surface an array of antibody-coated areas or spots.

-5-

Preferably, the support is a solid substance having a flat, planar surface such as a glass or plastic coverslip. The antibody-coated areas form small spots on the surface of the support surrounded by uncoated surface space. The antibodies in the antibody-coated spots are conjugated to the surface of the support; they may be either covalently or non-covalently bound thereto.

The most preferred arrangement of the spots on the surface of the support is a rectangular matrix. A matrix arrangement permits any spot to be readily identified by reference to coordinates giving the row and column number of the particular spot. The antibody spots should be closely spaced, thereby minimizing the surface space occupied by the complete arrangement of spots and in turn, minimizing the amount of sample needed. For instance, a 10 spot by 10 spot matrix should occupy about 1 cm<sup>2</sup> or less of the surface of the support.

In accordance with the invention, each spot, or each of certain sets of spots (such as the spots in one row or column), is made up of antibodies of single distinct specificity. Each spot, or each set of spots, contains antibodies whose specificity is different from the specificity of antibodies in the other spots. Thus in an 100 spot matrix 100 antibodies of different specificity may be tested. The antibody-coated spots serve as minute, specific immunoadsorbents for antigens with which the antibodies react. Antibody-coated spots made up of antibodies directed against a particular surface



-6-

antigen of cells serve as specific immunoadsorbents for cells bearing that surface antigen.

The density of antibodies in the antibody-coated spots is related to the function of the spots as immunoadsorbents of cells. The density of coated antibodies, either covalently or non-covalently conjugated to the surface of the support, must be such that the cells bind tightly to the spots and remain bound during ordinary manipulation of the device in subsequent procedures for detecting cell binding. It is suspected that bridges between the surface antigens of the cells and the antibodies attached to the surface of the support must be numerous in order to achieve tight cell binding. Preferably, however, the density of antibodies should be sufficient to yield, upon contact with an appropriate concentration of cells which have surface antigens that bind to the antibodies, a microscopically uniform layer of bound cells covering the entire antibody-coated spot. The formation of such a uniform layer of cells is desirable because it facilitates detection of spots to which cells have attached. The density required to generate a microscopically uniform layer of cells is probably greater than the density minimally sufficient for tight cell binding.

The immunoassay device of this invention is produced by applying small volumes of antibody solutions to the surface of the support and allowing the solutions to remain on the support until the antibodies become adsorbed to the surface. If



desired, the antibodies may be chemically bound to the surface of the support by employing conventional methods for covalently binding proteins to a solid phase.

5       The solutions of antibodies may be applied to the surface in the form of tiny droplets. The volume of the droplet, of course, determines the size of the antibody-coated area. Preferably, the volume should range between 0.005-0.5  $\mu$ l to yield  
10 antibody-coated dots with diameters of 0.25 mm - 1.0 mm. The droplets should be placed on the surface so as to conform to the desired arrangement of antibody-coated spots.

15       The solution can be delivered by micropipet, or by other suitable means. Preferably the applicator should permit application of droplets to the support surface in precise positions to cover uniform areas of substantially the same size. Any means for  
20 applying the solutions other than in the form of droplets, as for example, a device that makes direct contact with the surface of the support to transfer the antibody solution thereto, should meet the same requirements.

25       The preferred concentration of antibodies in the solution applied to the surface for coating is about 10  $\mu$ g/ml. It appears this is the optimal concentration for most antibodies. But because the binding of protein is dependent on its proportion in solution as well as its concentration, the solutions  
30 of antibodies must also be highly enriched in order to obtain a sufficiently dense antibody coat for the

-8-

desired cell adherence. Monoclonal antibodies can be obtained in adequately enriched solutions for producing the antibody-coated spots. As more monoclonal antibodies against cell surface antigens become available, antibody matrixes for the determination of a wide range of antigens can be devised. Also polyclonal antibodies may be obtained in adequately enriched solutions for the purposes of this invention.

After the solutions of various antibodies have been applied to the surface of the support and allowed to remain on the surface for the time necessary for adsorption or binding to occur, the entire surface of the support is flooded with a solution of protein or a solution of serum in order to dilute the unadsorbed antibodies and in order to block uncoated spaces and non-specific binding sites on the surrounding surface of the support. This protein solution is left on the surface for about 5 - 10 minutes. The device is then washed with distilled water and dried. The device may be used immediately, or it may be stored until use.

When the device is finished, antigen determinations can be performed in as little as 1 - 2 hours. Briefly, the method of using the device is as follows. Cells to be tested for the presence of certain surface antigens are placed in suspension in normal saline and applied to the surface of the device so that the cell entirely covers all the antibody-coated spots in the matrix. The number of cells needed to cover the entire matrix depends upon

-9-

the size of the matrix and the type of cell. Generally, however, approximately  $2 \times 10^6$  cells in a volume of 200  $\mu$ l are sufficient to cover all antibody-spots in a 1  $\text{cm}^2$  matrix of spots.

5        After the suspension of cells is applied to the surface of the support as above, the cells are allowed to settle to the surface and react with the antibodies in the spots. Next, non-adherent cells are removed from the surface. This can be accom-  
10       plished by washing and agitating the device or by any other suitable method which does not disrupt the adhered cells. The matrix of antibody spots is examined to determine to which spots cells have bound. Cells may be fixed and stained to aid in  
15       identifying the spots to which they have adhered.

      The immunoassay device of this invention can be used not only to determine the presence of a specific antigen on the surface cells, but also to analyze functionally different cell subpopulations  
20       that express distinct differentiation antigens. It has been established that an antibody-coated solid surface can adsorb quantitatively from a mixed population of cells the cells that bear the specific surface antigen with which the antibody react. The  
25       antibody matrix method of this invention could be used to determine the proportion of specific subsets in a mixed population, for example, the proportion of B cells, T cells, and monocytes in the mononuclear cell population or the proportion of inducer  
30       and suppressor T cells in the T cell population. Since the murine monoclonal antibodies that react

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-10-

specifically with all these cells are available, an immunoadherence device and method to accomplish the aforementioned may be developed. Although these tests can now be done with fluorescence flow cyto-

5 metry where each individual antibody analyzed separately, the antibody matrix method allows multiple determinations to be performed simultaneously.

Also, this invention is not limited to the

10 detection of antigens on the surface of cells. It is also applicable to the determination of soluble antigens, viruses, or bacteria. Some potential uses include the determination of the isotypes, allotypes or idiotypes of an antibody and the typing of a

15 viral or a bacterial sample.

The device and method of this invention may be adapted to the determination of the specificity of an antibody for antigen by employing an array or matrix of different antigen-coated spots, rather

20 than antibody-coated spots. The methodology for this type of determination would be essentially as described. A sample of antibody would be applied to the surface so as to come in contact with the entire array of antigen-coated spots and the binding of

25 antibody to the spots would be determined.

The following examples are intended to illustrate more fully the invention.

-11-

Examples

Monoclonal antibodies - Mouse anti-human HLA-A2 (IgG1), which was originally developed by Parham and Bodner, Nature 276:397 (1978), was purchased from Atlantic Antibodies, Scarborough, ME. The specific antibody had been enriched by the supplier from ascites fluid by a series of salt fractionation procedures. Allogeneic mouse anti-Lyt 2.1 (IgG<sub>2a</sub>) was originally developed by Shen and obtained from New England Nuclear, Boston MA in ascites fluid form. The IgG<sub>2a</sub> was enriched by absorbing it to protein A-Sepharose 4B column. Mouse anti-Lyt 2.2 (IgM) enriched from ascites fluid was a gift from Dr. David Raulet of the University of Pennsylvania. The original hybridoma was developed by Gottlieb et al., Immunogenetics 10:545 (1980).

Chemicals and specific glassware - Fluorescein isothiocyanate was purchased from Research Organics, Cleveland, Ohio. Pyrex disposable micro-sampling pipets (1-5 microliters) were from Corning Glass Works, Corning, NY. The pipets were heated and drawn into thinner pipets to deliver 0.005-1 ul. Graduations of 0.5 ul were marked by a fine-tip marker pen. Micro cover glasses (0.13 - 0.19 mm thick, 11 x 22 mm, 22 x 30 mm, and 22 x 40 mm) were obtained from Arthur H. Thomas Co. (catalog numbers 6663-F10, 663-K19, and 6663-K46).

Mice and cells - BALB/c mice were obtained from Charles River Breeding Laboratories, Wilmington, MA. AKR/J and (AKR/J x BALB/cJ) F1 mice were obtained from Jackson Laboratories, Bar Harbor, ME. These mice were 6-8 weeks old when used. Thymocytes were



-12-

prepared by teasing thymus through stainless screen. Human peripheral blood mononuclear cells were prepared by centrifuging heparinized blood on Ficoll/diatrizoate sodium solution ( $d=1.077$ ) (Pharmacia, Piscataway, NJ). Mononuclear cells were recovered from the interface of the 2-layer discontinuous density gradient.

Coating of antibodies on cover glass slips - A small piece of section paper (10 divisions in 1/2 inch length) was taped underneath the cover slip. The cover slips were placed at the center of a 10 cm diameter petri dish. The surrounding space in the dish and the inside of the lid were covered with wet paper tissue to retain moisture. Unless specified otherwise, about 0.05  $\mu$ l antibody solution was applied as a dot on the grid with a fine-tip 5  $\mu$ l micropipet. Antibodies were diluted to 10  $\mu$ g/ml with phosphate buffered saline, pH 7.4. Proper lighting was helpful for delivering droplets of uniform sizes. Application of droplets should be done in cold (4°C) room, if the air in the laboratory is dry. After incubation at room temperature for 60 min or at 4°C overnight, the paper tissue in the dish was removed and the cover slips were flooded swiftly with 30% calf serum to dilute the antibodies and to block uncoated space and binding sites. After 5-10 min, the cover slips were washed with distilled water and dried with an aspirator. The cover slips were immediately placed in petri dishes with the inside of the lids covered with wet paper tissue and the side of the dishes was sealed

-13-

with parafilm. The slips were then stored at 4°C until use.

Adhesion of cells to the antibody-coated glass slips - The antibody-coated slips were taped on petri dishes. Mouse thymocytes or human peripheral blood mononuclear cells were suspended at  $1 \times 10^7$ /ml in medium containing 1% fetal calf serum. A minimal suspension was placed on the antibody matrixes: about 50  $\mu$ l for a 5 x 5 matrix and 200  $\mu$ l for a 10 x 10 matrix. The cells were allowed to settle for 30 minutes at room temperature. About 10 ml phosphate buffered saline was added to the petri dish. Nonadherent cells were knocked off and flushed away by tapping the side of the dish 10-20 times. The retained cells on cover slips were then fixed by incubating in 10 ml formaldehyde solution (3.7%) for 30 min at room temperature. The cover slips were then washed with distilled water and air dried on paper tissue. In some cases, cells were further stained with fluorescein isothiocyanate (0.05 mg/ml in phosphate buffered saline) for 20 min at 37°C before washing with distilled water and drying.

Evaluation of cell binding - All the binding could be evaluated simply by the naked eye (without the use of the microscope). Certain samples were photographed on a black, smooth-surface, non fractile paper with 5 or 30 times magnification using a stereomicroscope.





-14-

Binding of cells to small areas of antibody-coated surface

Various amounts of mouse anti-human HLA-A2 antibody (10  $\mu\text{g/ml}$ ), ranging from approximately 5 0.005-0.5  $\mu\text{l}$ , were applied to small (11 x 22 mm) glass cover slips. Amounts larger than 0.05  $\mu\text{l}$  could be measured and delivered. The amount of solution in droplets smaller than 0.05  $\mu\text{l}$  was estimated by the number of droplets per unit volume 10 delivered. For example, if 20 droplets resulted from the delivery of 0.1  $\mu\text{l}$ , the average size of the droplets was 0.005  $\mu\text{l}$ . After the cover slips were prepared as described above, peripheral blood mononuclear cells isolated from individuals with 15 allotype A2 were applied to the antibody coated areas. After binding, nonadherent cells were washed away; adherent cells were fixed with formaldehyde and dried. Cover slips were examined with a regular microscope and photographed with a stereomicroscope. 20 Figure 1 shows the 1 mm, 0.5 mm, and 0.25 mm diameter dots at 50 times magnification. The glass surface is microscopically uniform.

Table 1 summarizes the cell-adsorbing capacity of antibody dots of varying sizes. The cover slips 25 were placed on a hemacytometer and total number of cells were estimated from the number of cells in a unit area and the diameter of the dots. The numbers probably represent the maximal capacity, since almost all space in the dots is filled. The small- 30 est 0.25 mm dots are still easily visible and can contain 1100 cells. These numbers should be within the limits that can be quantitated somehow with automatic analytical instruments.



-15-

Table 1. Number of cells in antibody dots of various sizes

	Size of dots (diameter)	1 mm	0.5 mm	0.25mm
5	Number of cells <sup>a</sup>	17000±2000	4500±300	1100±200

<sup>a</sup>Numbers are mean ± standard deviation of three determinations.

#### Analytical capacity of antibody matrixes

10 If antibodies of relevant but distinct specificities can be prepared and purified, they can be coated on a small area on a solid phase and used to analyze antigens. The most convenient organization of such antibody dots would appear to be arrays as exemplified in Fig. 2, in which one hundred antibody dots were arranged in a 10 x 10 "matrix" square. In 15 this example, mouse anti-human HLA-A2 was the antibody, and peripheral blood mononuclear cells from an individual with A2 allotype was the cell antigen.

20 Table 2 elucidates the analytical capacity of antibody matrixes. The discussion was based on a matrix area of 1 cm<sup>2</sup>. Assuming all antibodies are available, the typing for the HLA-A, B, C, and DR, which have about 80 allotypes altogether, can be



-16-

performed with one cell sample application. With the 0.25 mm dots, the tests of each individual allotypes can be repeated five times. It is apparent that the analysis of the cell binding results would be facilitated with proper instruments. Table 2 also indicates that each individual antibody matrix may have the capacity equivalent to one or several microtiter plates.

10 Table 2. The analytical capacity of using antibody matrixes of 1 cm<sup>2</sup> to determine antigens.

Size of dots (diameter)	1mm	0.5 mm	0.25 mm
Number of total dots <sup>a</sup>	25	100	400
15 Number equivalent of microtiter plates (60 wells)	0.5	1.5	7
20 Number of replicates for HLA typing (80 total allotypes)	-	1	5

<sup>a</sup>Distance between adjacent dots is equal to the diameter of the dots.

-17-

Cell sample and antibody requirement using antibody matrix analysis

Assuming mononuclear cells are 7  $\mu\text{m}$  in diameter (some are obviously larger), a square of 1  $\text{cm}^2$  may be completely covered with  $1.5 \times 10^6$  cells. In fact, in the present studies  $5 \times 10^5$  cells (in 50  $\mu\text{l}$ ) and  $2 \times 10^6$  cells (in 100  $\mu\text{l}$ ) were applied on areas of 0.25  $\text{cm}^2$  (5 x 5 matrix) and 1  $\text{cm}^2$  (10 x 10 matrix), respectively. Since one ml of blood contains on the average  $1 \times 10^6$  mononuclear cells, a few ml of blood is enough for a 1  $\text{cm}^2$  antibody matrix test.

The antibody matrix requires only very small amounts of reagents. If an antibody is coated at 10  $\mu\text{g/ml}$  concentration, which is optimal for most antibodies, 1 mg antibody can make 200,000 antibody dots of 0.5 mm diameter (see Table 3).



-18-

Table 3. Antibody solution required to coat antibody dots.

Size of dots (diameter)	1mm	0.5 mm	0.25 mm
5 Approximate volume antibody solution	0.5 $\mu$ l	0.05 $\mu$ l	0.005 $\mu$ l
Number of dots that 1 mg antibody can coat <sup>a</sup>	20,000	200,000	2,000,000

10 <sup>a</sup>Antibody concentration is 10  $\mu$ g/ml.

Binding of cells to specific antibody in the antibody matrix

15 Because the binding of a protein to a solid surface is dependent on not only its concentration but also its proportion in the solution, a specific antibody has to be rather enriched to be coated successfully. There are not many such antibodies available yet; the present studies use the antibodies that react with the allotypes of mouse T cell

20 surface differentiation antigen Lyt-2 to examine the specificity of cell binding to antibody dots in antibody matrix. Anti-Lyt 2.1 and Lyt 2.2

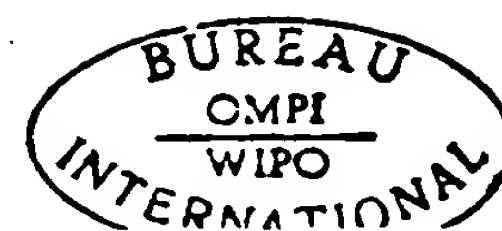
-19-

monoclonal antibodies have been developed, and isotypes of the antibodies can be readily isolated from ascites of mice bearing the specific antibody-producing hybridomas.

5 Fig. 3a shows schematically how the two antibodies are coated alternately to form 5 x 5 matrixes. The size of the matrixes are  $(0.5 \text{ cm})^2$  and the size of the dots is 0.5 mm in diameter. Thymocytes from AKR (Lyt 2.1<sup>+</sup>), BALB/c (Lyt 2.2<sup>+</sup>),  
10 and (AKR x BALB/c) F1 (Lyt 2.1<sup>+</sup> and 2.2<sup>+</sup>) were applied on the matrixes on a cover slip. Fig. 3b, 3c, and 3d show that AKR cells bind to only anti-Lyt 2.1 spots, BALB/c cells bind to only anti-Lyt 2.2 spots, and cells of their F1 bind to both spots.  
15 The binding is very specific. In a single cell binding assay using 5 x 5 matrix as shown in Fig. 3, the test with anti-Lyt 2.1 was repeated thirteen times and the test with anti-Lyt 2.2 twelve times.

## 20 Industrial Applicability

It is contemplated that this invention will be employed in clinical and research laboratories in tests or procedures requiring multiple antigen determinations. Generally, preparation of the  
25 immunoassay device, a procedure which requires the coating of a large number of antibodies onto glass coverslips or other types of supports, is not for routine practice in these laboratories. In order for this invention to be widely used, the devices  
30 should be made commercially available to users. To manufacture the devices in large number, a



-20-

mechanical applicator could be developed to apply the antibodies. As set forth above, the applicator should be able to precisely position the antibody-coated spots; also the applicator should be able to form spots of uniform shape (preferably round) and of substantially the same size.

For analysis of the results of cell binding to the antibody-coated spots, an automatic analyzer that would read cell binder to each antibody-coated spot and process this data is highly desirable.

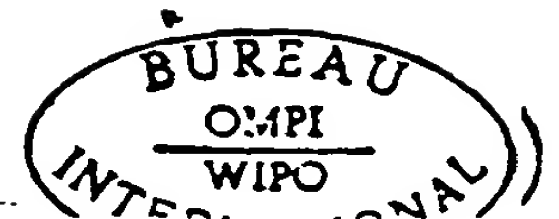
#### Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

-21-

CLAIMS

1. An immunoassay device comprising a support having on a surface thereof an array of small, discrete, closely spaced antibody-coated areas.
- 5 2. An immunoassay device according to Claim 1 wherein all of said antibody-coated areas have substantially the same size and shape.
- 10 3. An immunoassay device according to Claim 1, wherein the antibodies in said small, discrete antibody-coated surface areas are adsorbed to said surface uniformly and densely such that when cells bearing surface antigens to which said adsorbed antibodies bind are brought into contact with said antibody-coated areas in  
15 sufficient concentration, the cells adhere tightly to said antibody-coated area to form a layer of bound cells which is microscopically uniform over the entire antibody-coated area.
- 20 4. An immunoassay device according to Claim 1, wherein the antibodies in said antibody-coated areas are covalently bound to the surface of said support.
- 25 5. An immunoassay device according to Claim 1, wherein the antibodies in each of said antibody-coated surface areas, or in identifiable sets of said antibody-coated surface areas, are



-22-

antibodies of different and distinct specificity.

- 5 6. An immunoassay device according to Claim 1,  
wherein the antibodies in said antibody-coated  
areas are highly purified polyclonal anti-  
bodies.
7. An immunoassay device according to Claim 1,  
wherein the antibodies in said antibody-coated  
areas are monoclonal antibodies.
- 10 8. An immunoassay device according to Claim 1,  
wherein the support is a glass or plastic  
coverslip.
9. An immunoassay device according to Claim 1,  
wherein the array of said antibody-coated areas  
15 is a rectangular matrix.
10. An immunoassay device according to Claim 1,  
wherein said antibody-coated surface areas are  
dots having diameters of about 0.25 mm to 1.0  
mm.
- 20 11. An immunoassay device according to Claim 1,  
wherein said antibody-coated dots on the  
surface of the support are arranged in a  
compact 5 - 10 dot by 5 - 10 dot rectangular  
matrix.



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-23-

12. An immunoassay device according to Claim 10, wherein the entire rectangular matrix is contained within an area of about 0.5 - 1.0 cm<sup>2</sup> of the surface of the support.
- 5 13. An immunoassay device useful for determining allotypes of the Lyt 2 differentiation antigen on the surface of thymocytes comprising a glass coverslip having on its surface, small discrete anti-Lyt 2.1 and anti-Lyt 2.2 antibody-coated dots arranged in the form of a compact 5 dot by 5 dot rectangular matrix, said dots having diameters of 0.5 mm.
- 10
14. A method of producing an immunoassay device which comprises the steps of:
- 15 a) applying droplets of substantially enriched aqueous solutions of antibodies of distinct specificities to the surface of a support, locating said droplets so that they cover small, discrete, closely spaced areas of the surface of a support and form an array;
- 20 b) allowing said aqueous solutions of antibodies to remain on said areas of the surface for a period of time long enough for the antibodies in solution to be adsorbed to said areas of the surface to form antibody-coated areas;
- 25 c) swiftly flooding the entire surface of the solid support with an aqueous solution of protein in order to dilute the unadsorbed



-24-

antibodies in the various solutions of antibodies and in order to block non-specific binding sites in area of the surface of the solid support which surrounds the antibody-coated areas;

d) washing and drying the device.

15. A method of producing an immunoassay device according to Claim 14, wherein in step a said small, discrete areas to which said solutions of antibodies are applied are substantially the same in size and shape.

16. A method according to Claim 14, wherein in step a the concentration of antibodies in said aqueous solutions of antibodies is about 10  $\mu\text{g/ml}$ .

17. A method according to Claim 14, wherein in step a each solution of antibodies is applied to the surface of the solid support in the form of a tiny droplet having a volume of about 0.005  $\mu\text{l}$  - 0.5  $\mu\text{l}$ .

18. A method according to Claim 14, wherein said small, discrete areas of the surface of the support to which said solutions of antibodies are applied are dots having diameters of about 0.25 mm to 1.0 mm.

-25-

19. A method according to Claim 14, wherein said dots are located on the surface of the solid support to form a rectangular matrix.
20. A method of determining the presence of particular antigens on the surface of cells comprising the steps of:
- a) applying a suspension of cells to the surface of a support, said support having upon its surface an array of small, discrete, antibody-coated spots, each spot, or each of certain sets of spots, coated with antibodies of different and distinct specificity;
  - b) allowing the cells to settle upon the surface of the support so that the cells come into contact with all of the antibody-coated spots;
  - c) allowing the cells to react with the antibodies in the antibody-coated spots;
  - d) removing non-adherent cells;
  - e) determining to which antibody-coated spots the cells have adhered.
21. A method of determining allotypes of Lyt 2 differentiation antigen on the surface of thymocytes comprising the steps of:
- a) applying a suspension of thymocytes to the surface of a glass coverslip, said glass coverslip having upon its surface small, discrete anti-Lyt 2.1- and anti-Lyt 2.2-antibody-coated dots arranged in the form of a 5



-26-

dot by 5 dot rectangular matrix, so that the suspension covers the entire rectangular matrix of antibody-coated spots;

5        b) allowing the thymocytes to settle on the surface of the glass coverslip for about 30 minutes;

      c) removing non-adherent thymocytes by washing with phosphate buffered saline and agitating the coverslip;

10       d) staining the adherent cells with fluorescein isothiocyanate;

      e) visually inspecting the matrix of antibody-coated spots to determine whether the cells have adhered to the anti-Lyt 2.1-coated spots or the anti-Lyt 2.2-antibody-coated spots.

22. A method of determining the presence of particular antigens in a liquid comprising the steps of:

20       a) applying a sample of the liquid containing antigens to the surface of a support, said support having upon its surface an array of small, discrete antibody-coated spots, each spot coated with antibodies of different and distinct specificity, so that the antigen containing liquid comes into contact with all of the antibody-coated spots;

25       b) allowing the antigens to react with the antibodies in the antibody-coated spots;

-27-

c) washing the device to remove all unbound antigens;

d) determining to which antibody-coated spots antigens are bound.

5     23. A method of determining the proportion of cells bearing specific surface differentiation antigens in a mixed population of cells comprising:

10         a) applying a suspension of cells to the surface of a support, said support having upon its surface an array of small, discrete, antibody-coated spots, coated with antibodies of different and distinct specificity;

15         b) allowing the cells to settle upon the surface of the support so that the cells come into contact with all of the antibody-coated spots;

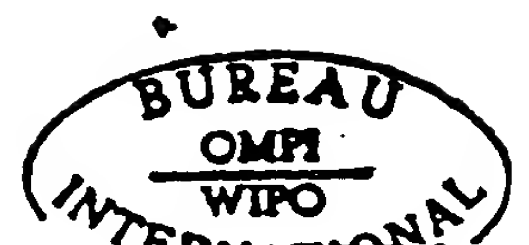
20         c) allowing the cells to react with the antibodies in the antibody-coated spots;

       d) removing non-adherent cells;

       e) quantitating cells bound to the antibody-coated spots; and

       f) calculating the relative proportion of cells.

25     24. An immunoassay device comprising a support having on a surface thereof an array of small, discrete, closely spaced antigen-coated areas each of said areas coated with a different antigen.



-28-

25. A method of determining the antigen specificity of antibodies in a liquid comprising the steps of:

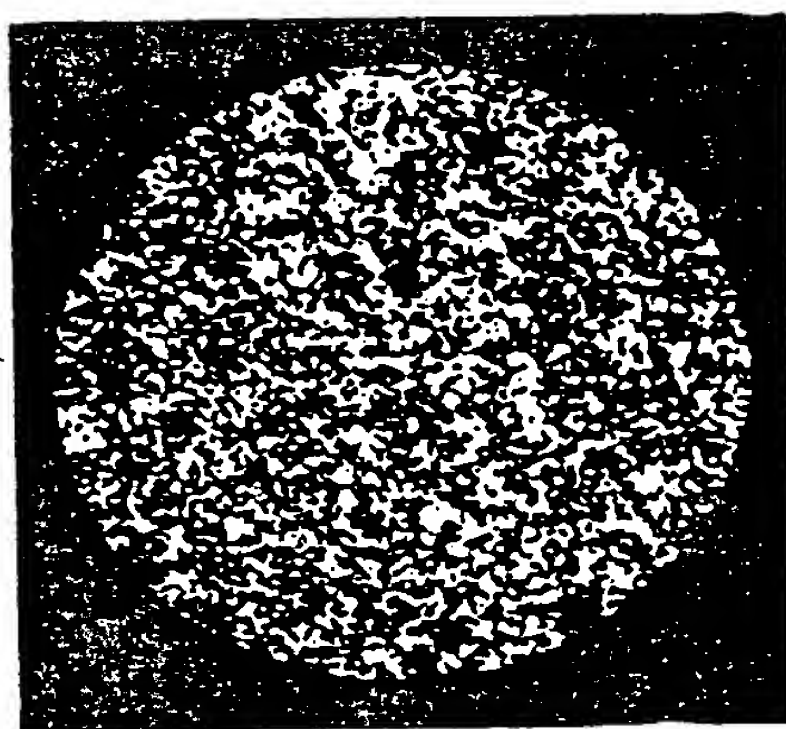
5 a) applying a sample of the liquid containing antibodies to the surface of a support, said support having upon its surface an array of small, discrete antigen-coated spots, each spot coated with a different antigen, so that the antibody containing liquid comes into contact with all of the antigen-coated spots;

10 b) allowing the antibodies to react with the antigens in the antigen-coated spots;

15 c) washing the device to remove all unbound antigens;

d) determining to which antigen-coated spots antibodies are bound.

1/8

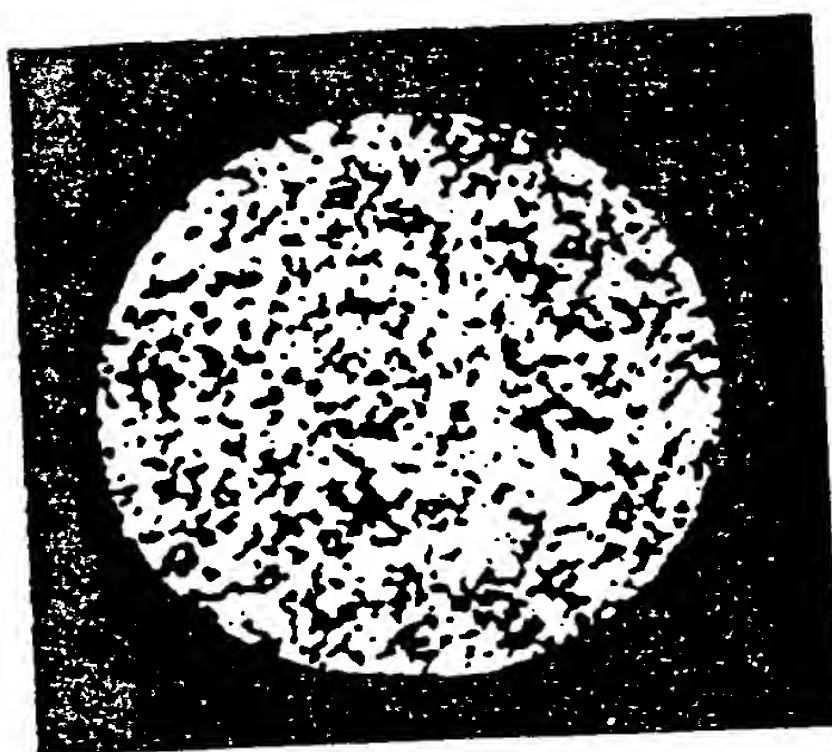


*Fig. 1A*

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2/8




***Fig. 1B***

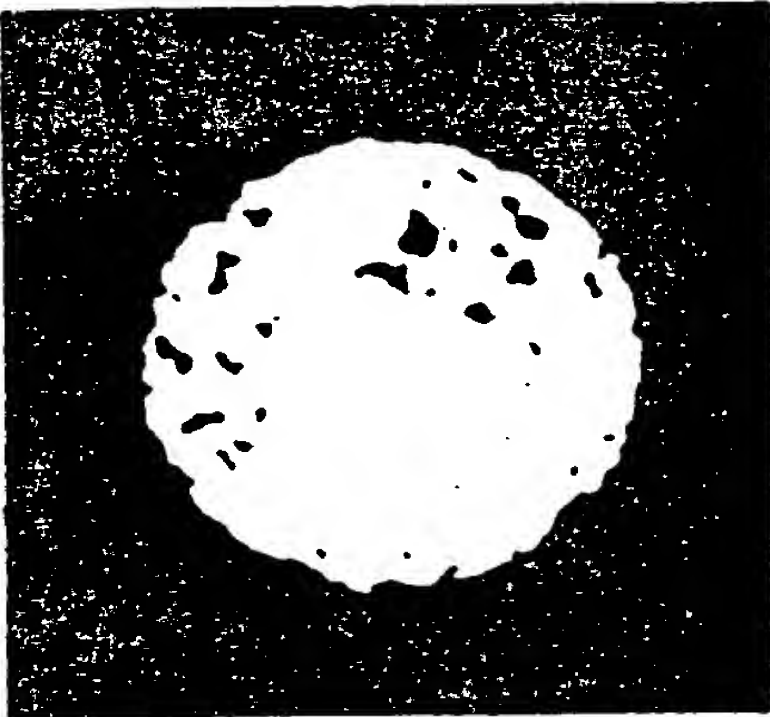
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3/8

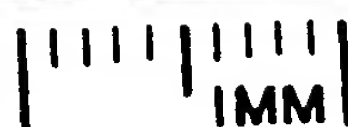
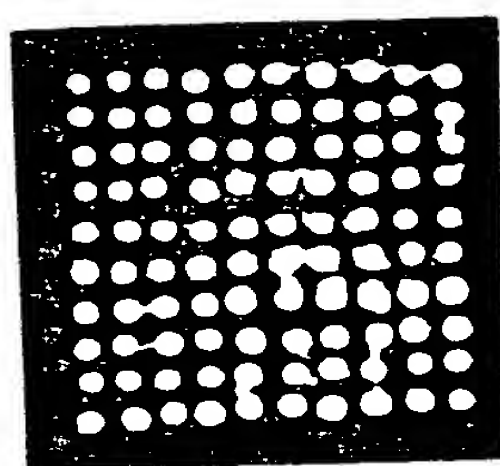


***Fig. 1C***

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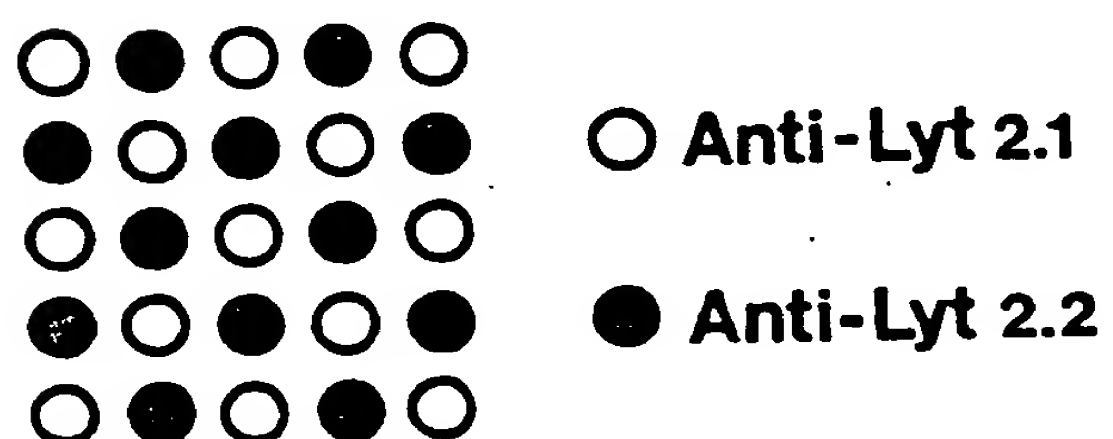
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*Fig. 2*

M I C R O S O F T

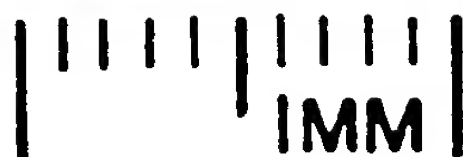
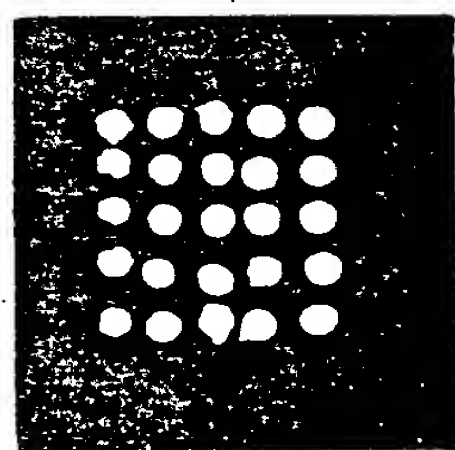
5/8

*Fig. 3A*

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6/8

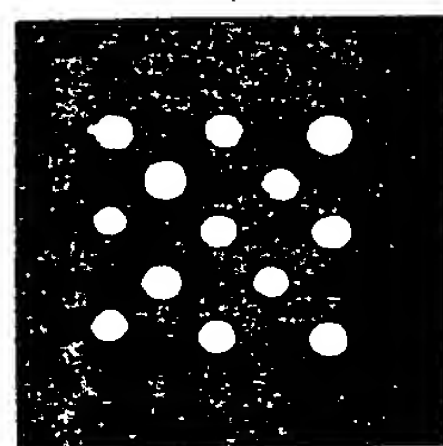


F 1  
*Fig. 3B*

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7/8



AKR  
*Fig. 3C*

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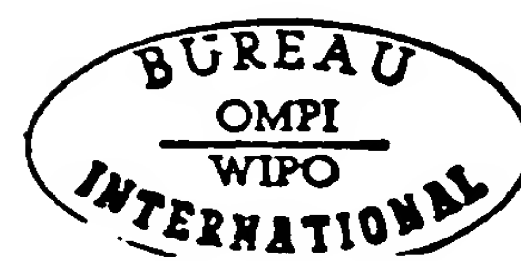
BUREAU  
OMPI  
WIPO

1. The first group of people who are not in the majority are those who are not in the majority in the majority.

IMM

BALB /c  
**Fig. 3 D**

**SUBSTITUTE SHEET**



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 84/00150

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>3</sup>: G 01 N 33/54

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
IPC <sup>3</sup>	G 01 N; A 61 K

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category *	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X, Y	EP, A, 0063810 (CIBA-GEIGY AG.) 3 November 1982 see the entire document	1-3, 5, 7-12, 14-25
Y	Chemical Abstracts, vol. 93, no. 9, 1 September 1980 (Columbus, Ohio, US) P.D. Gottlieb et al.: "Construction and properties of new lyt-congenic strains and anti-lyt-2.2 and anti-lyt-3.1 monoclonal antibodies", see page 481, column 1, abstract no. 93358f, Immunogenetics, 1980, 545-555 (Eng.) (cited in the application)	7, 21
A		13
Y	Chemical Abstracts, vol. 98, no. 11, 14 March 1983 (Columbus, Ohio, US) G.D. Johnson et al.: "Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy", see page 245, column 1, abstract no. 85548f, J. Immunol.	21 ./.

\* Special categories of cited documents: <sup>16</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search \*

10th May 1984

Date of Mailing of this International Search Report \*

06 JUIN 1984

International Searching Authority <sup>1</sup>

EUROPEAN PATENT OFFICE

Signature of Authorized Officer <sup>19</sup>

G.L.M. Kruijsen

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No <sup>18</sup>
------------	--	------------------------------------

	Methods, 1982, 231-242 (Eng.) --	
A	Nature, vol. 276, 23 November 1978, Macmillan Journals Ltd. (Basingstoke, GB) P. Parham et al.: "Monoclonal antibody to a human histocompatibility alloantigen, HLA-A2", see pages 397-399 (cited in the application) --	
A	US, A, 4046513 (L.C. JOHNSON) 6 September 1977 --	
A	US, A, 4331650 (J.H. BREWER et al.) 25 May 1982 --	
A	US, A, 3770383 (R.T. PRICE) 6 November 1973 --	
A	US, A, 4327073 (H.V. HUANG) 27 April 1982 --	
A	WO, A, 82/02601 (AMERICAN HOSPITAL SUPPLY CORPORATION) 5th August 1982 -----	



# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO.

PCT/US 84/00150 (SA 6555)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/05/84

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0063810	03/11/82	GB-A- 2099578 JP-A- 58009070 AU-A- 8306982	08/12/82 19/01/83 04/11/82
US-A- 4046513	06/09/77	LU-A- 77655 BE-A- 856280 NL-A- 7707274 FR-A, B 2356941 DE-A- 2729333 AT-A- 464177 AU-A- 2661377 JP-A- 53003885 AT-B- 352905 AU-B- 512788 CA-A- 1101319 SE-A- 7707539 CH-A- 637483	10/10/77 17/10/77 03/01/78 27/01/78 09/02/78 15/03/79 04/01/79 13/01/78 10/10/79 30/10/80 19/05/81 31/12/77 29/07/83
US-A- 4331650	25/05/82	None	
US-A- 3770383	06/11/73	US-A- 3666421	30/05/72
US-A- 4327073	27/04/82	None	
WO-A- 8202601	05/08/82	EP-A- 0070300	26/01/83